SKN-1 and GSK-3 Genes and Proteins

Related Applications

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Statement as to Federally Sponsored Research

This invention was made with Government support under National Institutes of Health Grant Nos. RO1GM50900 and RO1GM62891. The Government has certain rights in this invention.

Technical Field

This invention generally relates to the SKN-1 and GSK-3 genes and proteins, and their use in screening methods for isolating modulators of an oxidative stress response pathway.

Background

In diverse organisms, a common mesendodermal tissue field gives rise to the endoderm and a mesoderm subset that forms the heart and blood in vertebrates. In the nematode *Caenorhabditis elegans,* mesendodermal development is initiated by the maternally expressed transcription factor SKN-1, which specifies the fate of a single cell, the EMS blastomere. The EMS daughter cell E becomes the endoderm, which consists of the intestine. Its sister cell MS gives rise to mesodermal derivatives that include the pharynx, a feeding pump that is analogous to the heart, and coelomocytes that resemble macrophages. In these embryonic cells SKN-1 induces expression of the GATA factors MED-1 and MED-2, which are required for differentiation of EMS lineages. *C. elegans skn-1* mutants are sensitive to oxidative stress and have shortened lifespans.

In vertebrates, Nrf proteins activate transcription of genes encoding the Phase II detoxification enzymes, which constitute the primary cellular defense against oxidative stress. Essentially all organisms must defend themselves against reactive oxygen species (ROS), which are derived from both mitochondrial respiration and

exogenous sources. Phase II enzymes synthesize the critical reducing agent glutathione, scavenge ROS directly, and detoxify reactive intermediates that are generated when xenobiotics are metabolized by the cytochrome p450 (Phase I) enzymes. Through Nrf2, exposure to oxidative stress or particular classes of chemicals induces Phase II enzyme gene expression in a variety of tissues, including the liver and digestive tract. This mechanism also constitutes the major response to chemoprotective antioxidants, including many natural compounds, which thereby stimulate xenobiotic detoxification and inhibit carcinogen-induced tumorigenesis. Accordingly, mice that lack *Nrf2* are abnormally susceptible to drug toxicity and carcinogenesis, and do not respond to chemoprotective antioxidants.

Summary

The present invention is based in part on the discovery that SKN-1 is required for oxidative stress resistance and longevity in *C. elegans*. It has been discovered that SKN-1 orchestrates a major oxidative stress response in *C. elegans*, similar to Nrf proteins in vertebrates, in addition to initiating embryonic development of the *C. elegans* mesendoderm. The present invention is also based in part on the discovery that SKN-1 is modulated by GSK-3.

Accordingly, the present invention features in vivo and in vitro methods for determining whether a test compound is a candidate compound capable of modulating stress response, e.g., oxidative stress response, e.g., SKN-1-mediated oxidative stress response. For example, in one aspect the invention features methods for determining whether a test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound. In one embodiment, the method includes (a) contacting a GSK-3 polypeptide, or fragment thereof (e.g., an SKN-1 binding fragment), or a GSK-3 DNA with a test compound; (b) determining whether the test compound interacts with the GSK-3 polypeptide, or fragment thereof (e.g., SKN-1 binding fragment), or GSK-3 DNA; and (c) if the test compound interacts with the GSK-3 polypeptide, or fragment thereof (e.g., an SKN-1 binding fragment), or GSK-3 DNA, identifying the test compound as a candidate compound

capable of activating a stress response, e.g., an oxidative stress response, e.g., an SKN-1-mediated oxidative stress response.

In another embodiment, the method includes: (a) providing a GSK-3 polypeptide, or fragment thereof, e.g., a SKN-1 binding fragment thereof, and a SKN-1 polypeptide, or fragment thereof, e.g., a GSK-3 binding fragment thereof; (b) contacting the polypeptides or fragments thereof with a test compound; (c) determining whether the test compound decreases the interaction between the GSK-3 polypeptide, or fragment thereof, and the SKN-1 polypeptide, or fragment thereof, wherein a decrease in the interaction between the GSK-3 polypeptide, or fragment thereof, and the SKN-1 polypeptide, or fragment thereof, indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

In another embodiment, the method includes: (a) providing a nematode, e.g., a *C. elegans*, capable of expressing a SKN-1 polypeptide and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; and (b) contacting the nematode, e.g., a *C. elegans*, with a test compound; and (c) determining whether expression of the transgene is increased, wherein an increase in expression of the transgene indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second nematode, e.g., a second *C. elegans*, not contacted with the candidate compound, to determine whether the candidate compound increases the SKN-1-mediated oxidative stress response of the first nematode, e.g., *C. elegans*, relative to the SKN-1-mediated oxidative stress response of the second nematode,

e.g., the second *C. elegans*, not contacted with the candidate compound, wherein a candidate compound that increases SKN-1-mediated oxidative stress response in the first nematode relative to the second nematode is an SKN-1-mediated oxidative stress response-activating agent. In one embodiment, the confirmatory step includes providing a nematode, e.g., a *C. elegans*, not capable of expressing a SKN-1 polypeptide (e.g., a skn-1 mutant) and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; and contacting the nematode with the candidate compound, wherein no increase in expression of the transgene indicates that the candidate compound is a stress response-activating agent, e.g., an oxidative stress response-activating agent, e.g., an SKN-1-mediated oxidative stress response-activating agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of GSK-3 by: (a) contacting a GSK-3 polypeptide or GSK-3 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the GSK-3 polypeptide or GSK-3 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of GSK-3.

In another embodiment, the method includes: (a) providing a cell, e.g., a cultured cell, e.g., a cultured nematode or cultured mammalian cell, capable of expressing a SKN-1 polypeptide and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; and (b) contacting the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, with a test compound; and (c) determining whether expression of the transgene is increased, wherein an increase in expression of the transgene indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate

oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second cell, e.g., a second cultured cell, e.g., a second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound to determine whether the candidate compound increases the SKN-1-mediated oxidative stress response of the first cell, e.g., the first cultured cell, e.g., the first cultured nematode cell or first cultured mammalian cell, relative to the SKN-1-mediated oxidative stress response of the second cell, e.g., the second cultured cell, e.g., the second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound, wherein a candidate compound that increases SKN-1-mediated oxidative stress response in the first cell relative to the second cell is an SKN-1-mediated oxidative stress response-activating agent. In one embodiment, the confirmatory step includes providing a cell, e.g., a cultured cell, e.g., a cultured nematode cell or cultured mammalian cell, not capable of expressing a SKN-1 polypeptide (e.g., a skn-1 mutant cell) and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; and contacting the cell with the candidate compound, wherein no increase in expression of the transgene indicates that the candidate compound is a stress response-activating agent, e.g., an oxidative stress response-activating agent, e.g., an SKN-1-mediated oxidative stress response-activating agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of GSK-3 by: (a) contacting a GSK-3 polypeptide or GSK-3 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the GSK-3 polypeptide or GSK-3 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of GSK-3.

In another embodiment, the method includes: (a) providing a nematode, e.g., a *C. elegans*, containing a transgene encoding a SKN-1 fusion protein, wherein the transgene comprises: (i) a SKN-1 DNA operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; (b) contacting the nematode, e.g., *C. elegans*, with a test compound; and (c) determining whether the SKN-1 fusion protein accumulates in cell nuclei in the nematode, wherein increased accumulation indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second nematode, e.g., a second *C. elegans*, not contacted with the candidate compound to determine whether the candidate compound increases the SKN-1-mediated oxidative stress response of the first nematode, e.g., *C. elegans*, relative to the SKN-1-mediated oxidative stress response of the second nematode, e.g., the second *C. elegans*, not contacted with the candidate compound, wherein a candidate compound that increases SKN-1-mediated oxidative stress response in the first nematode relative to the second nematode is an SKN-1-mediated oxidative stress response-activating agent. In one embodiment, the confirmatory step includes providing a nematode, e.g., a *C. elegans*, not capable of expressing a SKN-1 polypeptide (e.g., a skn-1 mutant) and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; and contacting the nematode with the candidate compound, wherein no increase in expression of the transgene indicates that the candidate compound is a stress response-activating agent, e.g., an oxidative stress response-activating agent, e.g., an SKN-1-mediated oxidative stress response-activating agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of GSK-3 by: (a) contacting a GSK-3 polypeptide or GSK-3 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the GSK-3 polypeptide or GSK-3 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of GSK-3.

In another embodiment, the method includes: (a) providing a cell, e.g., a cultured cell, e.g., a cultured nematode or a cultured mammalian cell, containing a transgene encoding a SKN-1 fusion protein, wherein the transgene comprises: (i) a SKN-1 DNA operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; (b) contacting the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, with a test compound; and (c) determining whether the SKN-1 fusion protein accumulates in the nucleus of the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, wherein increased accumulation indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second cell, e.g., a second cultured cell, e.g., a second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound to determine whether the candidate compound increases the SKN-1-mediated oxidative stress response of the first cell, e.g., the first cultured cell, e.g., the first cultured nematode cell or first cultured mammalian cell, relative to the SKN-1-mediated oxidative stress response of the second cell, e.g., the second cultured cell, e.g., the second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound, wherein a candidate compound that increases SKN-1-mediated oxidative stress response in the first cell relative to the second cell is an SKN-1-mediated oxidative stress response-activating agent. In one embodiment, the confirmatory step includes providing a cell, e.g., a cultured cell, e.g., a cultured nematode cell or cultured mammalian cell, not capable of expressing a SKN-1 polypeptide (e.g., a skn-1 mutant cell) and containing at least one transgene including:

(i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; and contacting the cell with the candidate compound, wherein no increase in expression of the transgene indicates that the candidate compound is a stress response-activating agent, e.g., an oxidative stress response-activating agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of GSK-3 by: (a) contacting a GSK-3 polypeptide or GSK-3 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the GSK-3 polypeptide or GSK-3 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of GSK-3.

In another aspect, the invention features methods for determining whether a test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound. In one embodiment, the method includes (a) contacting a SKN-1 polypeptide, or fragment thereof, or a SKN-1 DNA with a test compound; (b) determining whether the test compound interacts with the SKN-1 polypeptide, or fragment thereof, or SKN-1 DNA; and (c) if the test compound interacts with the SKN-1 polypeptide, or fragment thereof, or SKN-1 DNA, identifying the test compound as a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In another embodiment, the method includes: (a) providing (i) a SKN-1 polypeptide, or fragment thereof, and (ii) an oxidative stress resistance gene, e.g., a gene encoding γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase, or SKN-1 polypeptide-binding fragments thereof; (b) contacting the SKN-1 polypeptide, or fragment thereof, and the oxidative stress resistance gene, or SKN-1

polypeptide-binding fragment thereof, with a test compound; and (c) determining whether the SKN-1 polypeptide, or fragment thereof, and the oxidative stress resistance gene, or SKN-1 polypeptide-binding fragment thereof, interact in the presence of the test compound, wherein a decrease in interaction indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In another embodiment, the method includes: (a) providing a nematode, e.g., a *C. elegans*, capable of expressing a SKN-1 polypeptide and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; (b) contacting the nematode, e.g., *C. elegans*, with a test compound; (c) before, during, or after step (b), subjecting the nematode, e.g., *C. elegans*, to conditions that activate the SKN-1-mediated oxidative stress response in the absence of the test compound; and (d) determining whether expression of the transgene, e.g., a transgene described herein, is decreased or unchanged, wherein decreased or unchanged expression of the transgene indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second nematode, e.g., a second *C. elegans*, not contacted with the candidate compound to determine whether the candidate compound decreases the SKN-1-mediated oxidative stress response of the first nematode, e.g., *C. elegans*, relative to the SKN-1-mediated oxidative stress response of the second nematode, e.g., the second *C. elegans*, not contacted with the candidate compound, wherein a candidate compound that decreases SKN-1-mediated oxidative stress response in the first nematode relative to the second nematode is a stress response-inhibiting agent,

e.g., an oxidative stress response-inhibiting agent, e.g., an SKN-1-mediated oxidative stress response-inhibiting agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of SKN-1 by: (a) contacting a SKN-1 polypeptide or SKN-1 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the SKN-1 polypeptide or SKN-1 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of SKN-1.

In another embodiment, the method includes: (a) providing a cell, e.g., a cultured cell, e.g., a cultured nematode or cultured mammalian cell, capable of expressing a SKN-1 polypeptide and containing at least one transgene including: (i) an oxidative stress resistance gene promoter, e.g., a promoter of a γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase gene, operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; (b) contacting the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, with a test compound; (c) before, during, or after step (b), subjecting the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, to conditions that activate the SKN-1-mediated oxidative stress response in the absence of the test compound; and (d) determining whether expression of the transgene, e.g., a transgene described herein, is decreased or unchanged, wherein decreased or unchanged expression of the transgene indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second cell, e.g., a second cultured cell, e.g., a second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound, to determine whether the candidate compound decreases the SKN-1-mediated oxidative stress response of the first cell, e.g., the first cultured cell, e.g., the first cultured nematode cell or first cultured mammalian cell, relative to the SKN-1-

mediated oxidative stress response of the second cell, e.g., the second cultured cell, e.g., the second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound, wherein a candidate compound that decreases SKN-1-mediated oxidative stress response in the first cell relative to the second cell is a stress response-inhibiting agent, e.g., an oxidative stress response-inhibiting agent, e.g., an SKN-1-mediated oxidative stress response-inhibiting agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of SKN-1 by: (a) contacting a SKN-1 polypeptide or SKN-1 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the SKN-1 polypeptide or SKN-1 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of SKN-1.

In another embodiment, the method includes: (a) providing a nematode, e.g., a *C. elegans*, containing a transgene encoding a SKN-1 fusion protein, wherein the transgene comprises: (i) a SKN-1 DNA operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; (b) contacting the nematode, e.g., *C. elegans*, with a test compound; (c) before or during step (b), subjecting the nematode, e.g., *C. elegans*, to conditions that activate the SKN-1-mediated oxidative stress response in the absence of the test compound; and (d) determining whether the SKN-1 fusion protein accumulates in cell nuclei in the nematode, wherein decreased or unchanged accumulation of the transgene indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second nematode, e.g., a second *C. elegans*, not contacted with the candidate compound, to determine whether the candidate compound decreases the SKN-1-mediated oxidative stress response of the first nematode, e.g., *C. elegans*, relative to the SKN-1-mediated oxidative stress response of the second nematode, e.g., the second *C. elegans*, not contacted with the candidate compound, wherein a candidate compound that decreases SKN-1-mediated oxidative stress response in the

first nematode relative to the second nematode is a stress response-inhibiting agent, e.g., an oxidative stress response-inhibiting agent, e.g., an SKN-1-mediated oxidative stress response-inhibiting agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of SKN-1 by: (a) contacting a SKN-1 polypeptide or SKN-1 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the SKN-1 polypeptide or SKN-1 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of SKN-1.

In another embodiment, the method includes: (a) providing a cell, e.g., a cultured cell, e.g., a cultured nematode or a cultured mammalian cell, containing a transgene encoding a SKN-1 fusion protein, wherein the transgene comprises: (i) a SKN-1 DNA operably linked to (ii) a reporter gene, e.g., a gene encoding green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase; (b) contacting the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, with a test compound; (c) before or during step (b), subjecting the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, to conditions that activate the SKN-1-mediated oxidative stress response in the absence of the test compound; and (d) determining whether the SKN-1 fusion protein accumulates in the nucleus of the cell, e.g., the cultured cell, e.g., the cultured nematode or cultured mammalian cell, wherein decreased or unchanged accumulation of the transgene indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In one embodiment, the method further includes a confirmatory step carried out after a candidate compound is identified. The confirmatory step can include providing a second cell, e.g., a second cultured cell, e.g., a second cultured nematode cell or second cultured mammalian cell, not contacted with the candidate compound to determine whether the candidate compound decreases the SKN-1-mediated oxidative stress response of the first cell, e.g., the first cultured cell, e.g., the first cultured nematode cell or first cultured mammalian cell, relative to the SKN-1-mediated oxidative stress response of the second cell, e.g., the second cultured cell, e.g., the second cultured nematode cell or second cultured mammalian cell, not

contacted with the candidate compound, wherein a candidate compound that decreases SKN-1-mediated oxidative stress response in the first cell relative to the second cell is a stress response-inhibiting agent, e.g., an oxidative stress response-inhibiting agent, e.g., an SKN-1-mediated oxidative stress response-inhibiting agent.

In one embodiment, the method further includes determining whether the candidate compound is an inhibitor of SKN-1 by: (a) contacting a SKN-1 polypeptide or SKN-1 DNA with the candidate compound; and (b) detecting interaction of the candidate compound with the SKN-1 polypeptide or SKN-1 DNA, wherein an interaction indicates that the candidate compound is an inhibitor of SKN-1.

In another aspect, the invention features a method of modulating stress response, e.g., oxidative stress response, e.g., SKN-1-mediated oxidative stress response, by inhibiting GSK-3. In one embodiment, stress response, e.g., oxidative stress response, e.g., SKN-1-mediated oxidative stress response, is modulated by a compound that interacts with GSK-3, e.g., a compound described herein.

In another aspect, the invention features fusion proteins, e.g., SKN-1 fusion proteins, nucleic acids encoding fusion proteins, e.g., SKN-1 fusion proteins, vectors containing nucleic acids encoding fusion proteins, e.g., SKN-1 fusion proteins, and cells, e.g., cultured cells, e.g., cultured nematode or cultured mammalian cells, expressing fusion proteins, e.g., SKN-1 fusion proteins. In one embodiment, the fusion protein, e.g., the SKN-1 fusion protein, includes an SKN-1 polypeptide, or a fragment thereof, e.g., a biologically active fragment thereof, and a heterologous polypeptide, e.g., a reporter polypeptide, e.g., green fluorescent protein, chloramphenicol acetyl transferase, ß glucuronidase, or luciferase.

In another aspect, the invention features transgenic nematodes, e.g., transgenic *C. elegans*, expressing a transgene. In one embodiment, one or more cells of the transgenic nematode, e.g., transgenic *C. elegans*, express a transgene, e.g., a transgene that encodes a fusion protein, e.g., a protein described herein fused to a reporter gene described herein, e.g., an skn-1::gfp transgene, a gcs-1::gfp transgene, or a gsk-3::gfp transgene.

In another aspect, the invention features compounds capable of activating an SKN-1-mediated oxidative stress response isolated using the methods described herein (e.g., for isolating SKN-1 mediated oxidative stress response inhibitors or activators). In one embodiment, the compounds, e.g., compounds described herein, interact with GSK-3. In one embodiment, a test compound that interacts with GSK-3, e.g., a test compound described herein, is identified as a candidate compound that can manipulate stress response, e.g., oxidative stress response, e.g., SKN-1-mediated oxidative stress response.

The invention also features the use of a compound identified herein in the manufacture of a medicament for treatment or prevention of a condition described herein. The medicament can be in any form described herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although suitable methods and materials for the practice or testing of the present invention are described below, other methods and materials similar or equivalent to those described herein, which are well known in the art, can also be used. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Description of the Drawings

Figs. 1A-1C: SKN-1 embryonic functions and comparison to Nrf proteins. 1A: Cell fate specification. In four-cell embryos, SKN-1 initiates mesendodermal development by establishing the EMS blastomere fate. The anterior pharynx is specified in ABa descendants by an SKN-1-dependent signal from MS. Anterior is to the left, and ventral at the bottom. 1B: SKN-1 compared to Nrf proteins. The SKN-1

minor groove-binding arm is shown in light green. Percent identity between SKN-1 and mouse Nrf2 regions is indicated. 1C: Consensus sequences for SKN-1 binding and the ARE. The SKN-1 BR recognizes a consensus bZIP half-site (underlined) adjacent to an AT-rich motif (grey) that is specified by the arm (B). Nrf proteins bind to the ARE as obligate heterodimers with Maf or other bZIP. R=G/A; W=T/A.

Figs. 2A-2L: *skn-1*-dependent GCS-1::GFP expression in the intestine and ASI neurons. 2A-2F: GCS-1::GFP expression in wild type animals. A *gcs-1* genomic fragment containing its 17 amino terminal codons and 1840 upstream bp was fused to the amino terminus of GFP that contained a nuclear localization signal. Expression patterns shown are each representative of more than two independent transgenic lines, and of all postembryonic stages examined (L2- adult; data not shown). 2A and 2B show Nomarski and fluorescent views, respectively, of an L2 larva. In 2B a line demarcates the approximate boundary between the anterior intestine (I) and posterior pharynx (P). 2C and 2D show combined Nomarski/fluorescent and fluorescent views, respectively, of the head of a typical L4 stage animal that had been exposed to DiI. In 2D one of the two ASI neurons is indicated with an arrow. 2E and 2F show an L2 larva in which GCS-1::GFP expression was induced to high levels in the intestine by heat. A similar induction occurred in response to paraquat (Table 2). The boundary between the anterior intestine and posterior pharynx is indicated as in 2B. 2G-2L: GCS-1::GFP was not detectable outside of the pharynx in *skn-1* homozygotes. Typical animals are shown from experiments that parallel those displayed to the left in 2A-2F. Note the absence of GCS-1::GFP in the intestine and ASI neurons under normal conditions 2G-2J, and after treatment with heat (2K, 2L) or paraquat (data not shown). In two independent transgenic lines, in a homozygous *skn-1* background GCS-1::GFP expression was not detected in these tissues in any animals under either normal or induction conditions.

Figs. 3A-3C: Specific elements required for *skn-1*-independent and -dependent GCS-1::GFP expression. 3A: Analysis of the *gcs-1* promoter. Expression of the indicated constructs from transgenic extrachromosomal arrays was assayed in 2-3 independent transgenic lines, under normal conditions and after induction by paraquat and heat. Approximate relative expression levels in the tissues designated to the right (data not shown) are indicated by + signs, with ++ indicating a reproducible

reduction, and + indicating barely detectable expression. Within each set of transgenic lines that carried promoter mutations, levels of normal and induced expression were affected in parallel. Mutations that were created in predicted SKN-1 sites 1, 2, and 3 are described in Materials and Methods, and are not compatible with SKN-1 binding (see text). Red ovals indicate predicted SKN-1 binding sites and a green bar the 5' end of the *gcs-1::gfp* coding region. Map numbers refer to the predicted translation start. 3B: Uncoupling pharyngeal GCS-1::GFP expression from intestinal and ASI neuron expression. The *gcsΔ 2* mutation eliminated pharyngeal GCS-1::GFP expression, but allowed near-wild type levels of ASI and intestinal expression. Concurrent ablation of SKN-1 binding site 3 (*gcsΔ 2,mut3*) eliminated transgene expression in all tissues. Paraquat-treated worms are shown in the GFP column. 3C: Composite *gcs-1* promoter element that includes SKN-1 site 3, and is also present in the *med-1* and *-2* promoters. SKN-1 binding sites are red, and identical sequences are boxed.

Figs. 4A and 4B: Specific binding of SKN-1 to an essential *gcs-1* promoter sequence. 4A: Binding of full-length SKN-1 to site 3 within the *gcs-1* composite element, assayed by EMSA. Lanes 2-5 show binding of increasing amounts of *in vitro* translated SKN-1 protein (0 µl, 0.25 µl, 0.5 µl, 3 µl translation lysate; indicated by a triangle) to the wild type site. Lane 1 shows binding to 3 µl unprogrammed lysate. A background species is labeled. Lanes 6-10 show the same assay performed with the mutant probe. In lanes 11-20, SKN-1-DNA binding is assayed in the presence of the indicated unlabelled competitor oligonucleotides. Lanes 12-15 and 17-20 correspond to addition of a 20-, 50-, 150-, and 400-fold molar excess of competitor over the labeled wild-type DNA. 4B: The *in vitro* translated SKN-1 DNA binding domain (Fig. 1B) binds specifically to the *gcs-1* composite element. Binding was assayed as in 4A.

Figs. 5A-5G: Expression and stress-induced nuclear accumulation of SKN-1::GFP. 5A: SKN-1::GFP transgenes **a.** *skn-1* gene. Transcribed coding and untranslated regions are indicated in red and blue, respectively. **b.** SKN-1::GFP translational fusion construct, which includes an EcoR1 fragment that previously rescued maternal *skn-1* lethality. *C. elegans* DNA is indicated by a black line. **c.** SknPro::GFP promoter fusion, in which the 38 N-terminal SKN-1 amino acids are

fused to GFP containing a nuclear localization signal. 5B-5D: Embryonic expression of SKN-1::GFP. 5B, 5C, and 5D show Nomarski (left) and fluorescent (right) views, of 100 cells, 280 min., and three fold embryos, respectively. Endogenous intestinal autofluorescence is visible as yellow or orange. White triangles indicate intestine precursor nuclei. Int: intestine. Ph: pharynx. 5E: SKN-1::GFP expression in ASI neurons (arrows). Nomarski/fluorescent (left) and fluorescent (right) views are shown of a typical DiI-exposed L4 larva. 5F: Larval SKN-1::GFP expression under normal conditions. Fluorescent and Nomarski closeups of the boxed region of this L2 are shown at bottom. Note the low-level SKN-1::GFP expression in intestinal nuclei (white triangle). 5G: SKN-1::GFP localization under oxidative stress. Examination of multiple focal planes revealed that SKN-1::GFP levels were not substantially altered in ASI neurons (arrows), but in many animals were dramatically increased in intestinal nuclei (Table 3). A heat-shocked L2 is shown, but similar results were obtained upon exposure to other oxidative stress inducers (Table 3). The integrated strain *Is007* is shown, but two extrachromosomal lines and a different integrated line exhibited similar patterns.

Figs. 6A-6B: *skn-1* mutants are sensitive to oxidative stress and have reduced lifespans. 6A: Paraquat sensitivity. Worms were scored for survival at the times shown after they had been placed in M9 that contained 100 mM paraquat. An average of three experiments involving 24 worms each is graphed. All wild type and *skn-1* mutant worms survived a parallel control 72 hr. incubation in M9 alone (data not shown). 6B: Lifespan assay. Worms were maintained at 20°C and scored for survival at the indicated time after the L4 stage. An average of three experiments involving 25-28 worms each is plotted. In wild type, *skn-1(zu67)*, and *skn-1(zu129)* strains, mean life spans were 15.9+2.2, 11.8+1.4, and 11.1+0.1 days, respectively. Mean maximum life spans were 24.3+3.5, 16.3+0.6, and 18.7+0.6 days, respectively.

Fig. 7 illustrates an exemplary regulatory sequence for the glutathione synthetase gene.

Fig. 8 illustrates the sequences of the glutathione synthetase ORF and protein.

Fig. 9 illustrates an exemplary regulatory sequence for the NADH quinone oxidoreductase gene.

Fig. 10 illustrates the sequences of the NADH quinone oxidoreductase ORF and protein.

Fig. 11 illustrates an exemplary regulatory sequence for the glutathione S-transferase (R03D7.6) gene.

Fig. 12 illustrates the sequences of the glutathione S-transferase (R03D7.6) ORF and protein.

Fig. 13 illustrates an exemplary regulatory sequence for the glutathione S-transferase (F35E8.8) gene.

Fig. 14 illustrates the sequences of the glutathione S-transferase (F35E8.8) ORF and protein.

Fig. 15 illustrates an exemplary regulatory sequence for the glutathione S-transferase (F11G11.2) gene.

Fig. 16 illustrates the sequences of the glutathione S-transferase (F11G11.2) ORF and protein.

Fig. 17 illustrates an exemplary regulatory sequence for the glutathione S-transferase (K08F4.7) gene.

Fig. 18 illustrates the sequences of the glutathione S-transferase (K08F4.7) ORF and protein.

Fig. 19 illustrates an exemplary regulatory sequence for the superoxide dismutase-1 (*sod-1*) gene.

Fig. 20 illustrates the sequences of the superoxide dismutase-1 (*sod-1*) ORF and protein.

Fig. 21 illustrates an exemplary regulatory sequence for the superoxide dismutase-2 (*sod-2)* gene.

Fig. 22 illustrates the sequences of the superoxide dismutase-2 (*sod-2)* ORF and protein.

Fig. 23 illustrates an exemplary regulatory sequence for the catalase (*ctl-1)* gene.

Fig. 24 illustrates the sequences of the catalase (*ctl-1)* ORF and protein.

Fig. 25 illustrates an exemplary regulatory sequence for the superoxide dismutase-3 (*sod-3)* gene.

Fig. 26 illustrates the sequences of the superoxide dismutase-3 (*sod-3)* ORF and protein.

Fig. 27 illustrates an exemplary regulatory sequence for the γ-glutamine cysteine synthase (also known as glutamate-cysteine ligase) heavy chain gene.

Fig. 28 illustrates the sequences of the γ-glutamine cysteine synthase (also known as glutamate-cysteine ligase) heavy chain open reading frame (ORF) and protein.

Fig. 29 illustrates the sequences of the T19E7.2c SKN-1 ORF and protein.

Fig. 30 illustrates the sequences of the T19E7.2b SKN-1 ORF and protein.

Fig. 31 illustrates the sequences of the T19E7.2a SKN-1 ORF and protein.

Fig. 32 illustrates the amino acid sequence of human GSK-3 beta.

Fig. 33 illustrates the amino acid sequence of human GSK-3 alpha.

Fig. 34 illustrates the amino acid sequence of mouse GSK-3 beta.

Fig. 35 illustrates the amino acid sequence of mouse GSK-3 alpha.

Fig. 36 illustrates the amino acid sequence of *C. elegans* GSK-3.

Fig. 37 is an image of (A) SKN-1::GFP localization and (B) GCS-1::GFP localization.

Fig. 38 is (A) a schematic of predicted phosphorylation sites in SKN-1 and (B) an image of mutant SKN-1::GFP localization.

Fig. 39 is (A) a schematic of predicted phosphorylation sites and (B) a graph of phosphorylation of various SKN-1 peptides by GSK-3.

Detailed Description

The present invention relates, in part, to the *C. elegans* SKN-1 gene and protein (a transcription factor), the GSK-3 gene and protein, *gcs-1* (encoding γ-glutamine cysteine synthase heavy chain (GCS(h); a target gene of SKN-1) and other oxidative stress resistance genes, e.g., M176.2 (encoding glutathione synthetase); F39B2.3 (encoding NADH quinone oxidoreductase); *sod-1, sod-2,* and *sod-3* (encoding superoxide dismutase); *ctl-1 (encoding* catalase); and R03D7.6, F35E8.8, F11G11.2, and K08F4.7 (encoding glutathione S-transferase). The invention includes various therapeutic and screening methods. For example, the genes and/or proteins described herein can be used in screening methods to identify compounds, e.g.,

compounds capable of modulating (e.g., increasing or decreasing) the expression and/or activity of SKN-1 or the expression and/or activity of GSK-3, and/or capable of modulating the oxidative stress response pathway in nematodes and other animals (e.g., humans). Such compounds can be used as pharmaceutical agents and/or pesticides.

Before further description of the invention, certain terms employed in the specification, examples, and appended claims are, for convenience, collected here.

By "SKN-1-mediated oxidative stress response" is meant an oxidative stress response pathway mediated (i.e., activated) by SKN-1 polypeptides.

The *C. elegans* genome has been sequenced (see, e.g., The *C. elegans* Sequencing Consortium, Science 282, p.2012-2018, 1998), and is accessible through several known electronic databases (see, e.g., the databases accessible at World Wide Web (www) addresses: wormbase.org (WormBase; see, Harris et al., Nucleic Acids Research 31:133-137 (2003), and Stein et al., Nucleic Acids Research 29:82-86 (2001)); ncbi.nlm.nih.gov; and wormbase.sanger.ac.uk). "SKN-1 DNA" or "SKN-1 gene" refers to nucleic acid sequences that include, e.g., the nucleic sequence set forth in Fig. 29 (or the unspliced version thereof) (set forth in the WormBase database as T19E7.2c) and/or Fig. 31 or 30 (or the unspliced versions thereof) (set forth in the WormBase database as T19E7.2a, and T19E7.2b, respectively), homologs thereof, or fragments thereof that encodes SKN-1 polypeptide fragment capable of binding a SKN-1 protein binding site within a promoter of a target gene, e.g., a *C. elegans* Phase II detoxification gene. An example of such a fragment is a fragment that encodes the C-terminal 85 amino acid residues of the SKN-1 polypeptide set forth in Fig. 29 (referred to herein as a "SKN-1 Domain"). By "SKN-1 polypeptide" is meant an amino acid sequence that includes an amino acid sequence set forth in Figs. 29, 30 and/or 31, or fragments thereof (e.g., the C-terminal 85 amino acid residues of the SKN-1 polypeptide set forth in Fig. 29 (a "SKN-1 Domain), or amino acids 381-403 set forth in Fig. 29, or amino acids 473-495 set forth in Fig. 31). By "SKN-1 RNA" is meant messenger RNA transcribed from a SKN-1 DNA sequence.

"GSK-3 DNA" or "GSK-3 gene" refers to nucleic acid sequences encoding GSK-3 (such sequences are known in the art), homologs thereof, or fragments thereof, that encode GSK-3 polypeptide fragments capable of binding a SKN-1 protein. By

"GSK-3 polypeptide" is meant an amino acid sequence that includes an amino acid sequence set forth in Figs. 32, 33, 34, 35 and/or 36, or fragments thereof. By "GSK-3 RNA" is meant messenger RNA transcribed from a GSK-3 DNA sequence.

As used herein, an "oxidative stress resistance gene" is a gene involved in the oxidative stress response in *C. elegans*, and homologs thereof. For example, oxidative stress resistance genes include those demonstrated herein to be targets of the SKN-1 polypeptide, e.g., those encoding γ-glutamine cysteine synthase heavy chain (GCS(h)) (*gcs-1*, set forth in WormBase as F37B12.2); glutathione synthetase (set forth in WormBase as M176.2); NADH quinone oxidoreductase (set forth in WormBase as F39B2.3); superoxide dismutase (*sod-1, sod-2,* and *sod-3*; set forth in WormBase as C15F1.7; F10D11.1; C08A9.1, respectively); catalase (*ctl-1*; set forth in WormBase as Y54G11A.6; and several forms of glutathione S-transferase (set forth in WormBase as R03D7.6, F35E8.8, F11G11.2, and K08F4.7), among others.

The sequence of the γ-glutamine cysteine synthase (also known as glutamate-cysteine ligase) heavy chain open reading frame (ORF) and protein are set forth in Fig. 28, and an exemplary regulatory sequence is set forth in Fig. 27. The sequence of the glutathione synthetase ORF and protein are set forth in Fig. 8, and an exemplary regulatory sequence is set forth in Fig. 7. The sequence of the NADH quinone oxidoreductase ORF and protein are set forth in Fig. 10, and an exemplary regulatory sequence is set forth in Fig. 9. The sequence of one glutathione S-transferase (R03D7.6) ORF and protein are set forth in Fig. 12, and an exemplary regulatory sequence is set forth in Fig. 11. The sequence of another glutathione S-transferase (F35E8.8) ORF and protein are set forth in Fig. 14, and an exemplary regulatory sequence is set forth in Fig. 13. The sequence of a third glutathione S-transferase (F11G11.2) ORF and protein are set forth in Fig. 16, and an exemplary regulatory sequence is set forth in Fig. 15. The sequence of a fourth glutathione S-transferase (K08F4.7) ORF and protein are set forth in Fig. 18, and an exemplary regulatory sequence is set forth in Fig. 17. The sequence of the superoxide dismutase-1 (*sod-1*) ORF and protein are set forth in Fig. 20, and an exemplary regulatory sequence is set forth in Fig. 19. The sequence of the superoxide dismutase-2 (*sod-2)* ORF and protein are set forth in Fig. 22 and an exemplary regulatory sequence is set forth in Fig. 21. The sequence of the superoxide dismutase-3 (*sod-3)* ORF and protein are set

forth in Fig. 26, and an exemplary regulatory sequence is set forth in Fig. 25. The sequence of the catalase (*ctl-1)* ORF and protein are set forth in Fig. 24 and an exemplary regulatory sequence is set forth in Fig. 23. Predicted SKN-1 binding sites upstream of the genes described above are set forth in Table 1.

A "substantially pure" preparation is a preparation that is at least 60% by weight (dry weight) compound of interest. For example, a "substantially pure" preparation can be a preparation that is at least 60% by weight of SKN-1 polypeptide, GSK-3 polypeptide, or a candidate compound or agent described herein. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

The term "purified DNA" means DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism from which it is derived. The term, therefore, includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

A "substantially identical" nucleic acid means a nucleic acid sequence that encodes a polypeptide differing only by conservative amino acid substitutions or by one or more non-conservative substitutions, deletions, or insertions located at positions of the amino acid sequence that do not destroy the function of the polypeptide.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains

(e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue can be replaced with another amino acid residue from the same side chain family.

The terms "activate," "induce," "inhibit," "elevate," "increase," "decrease," or the like, denote quantitative differences between two states. A quantitative difference can be, e.g., a statistically significant difference, between the two states.

Homology can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, or PILEUP/PRETTYBOX programs). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

By "purified antibody" is meant antibody that is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody.

The term "longevity" refers to the rate of senescence and/or life-span. The term "immunological methods" refers to any assay involving antibody-based detection techniques including, without limitation, Western blotting, immunoprecipitation, and direct and competitive ELISA and RIA techniques. "Means for detecting" refers to any one or a series of components that sufficiently indicate a detection event of interest. Such means involve at least one label that may be assayed or observed, including, without limitation, radioactive, fluorescent, and chemiluminescent labels.

By "hybridization techniques" is meant any detection assay involving specific interactions (based on complementarity) between nucleic acid strands. Such interactions include, e.g., DNA-DNA, RNA-RNA, and DNA-RNA interactions. Such hybridization techniques may, if desired, include a PCR amplification step.

The term "modulatory compound", as used herein, refers to any compound capable of either increasing or decreasing SKN-1 expression (i.e., at the level of transcription or translation), SKN-1 polypeptide activity, GSK-3 expression, GSK-3 polypeptide activity, or activating or inhibiting a stress response pathway, e.g., an oxidative stress response pathway, e.g., a SKN-1-mediated oxidative stress response pathway.

The terms "effective amount" and "effective to treat," as used herein, refer to an amount or concentration of a pharmaceutical composition described herein utilized for a period of time that is effective within the context of its administration for causing an intended effect or physiological outcome. The period of time includes acute or chronic administration and periodic or continuous administration.

The term "patient" refers to an animal, human or non-human, to whom treatment according to the methods of the present invention is provided. Veterinary applications are clearly anticipated by the present invention. The term includes, but is not limited to, birds, reptiles, amphibians, and mammals, e.g., humans, other primates, pigs, rodents (such as mice and rats), rabbits, guinea pigs, hamsters, cows, horses, cats, dogs, sheep and goats. Preferred subjects are humans, farm animals, and domestic pets such as cats and dogs. The terms "treat" and "treatment" are used herein to denote the use of a protein, agent, or composition described herein to delay the onset of, to inhibit, to alleviate the effects of, or to prolong the life of a patient.

Screening Methods

In vertebrates, the Nrf pathway has been shown to be critical for oxidative stress resistance, and to be involved in numerous disease states including diabetes, HIV infection, and atherosclerosis, as well as in drug detoxification and cancer chemoprevention. The vertebrate Nrf pathway responds to natural antioxidants found in plants, which produce these compounds for protection against errors in photosynthesis. The present invention is based in part on the discovery that, in addition to its role in development, SKN-1 plays a role in the *C. elegans* counterpart to the vertebrate Nrf pathway. Like the Nrf pathway, the *C. elegans* pathway mediated by SKN-1 responds to the well-studied natural antioxidant sulforaphane.

GSK-3 (Glycogen synthase kinase 3) was initially described as a key enzyme involved in glycogen metabolism, but is now known to regulate a diverse array of cell functions. See, e.g., Cohen et al. (2001) *Mol. Cell Biol.* 2:769-776.

The invention provides *in vivo* and *in vitro* screening methods for identifying compounds, e.g., small organic or inorganic molecules (M.W. less than 1,000 Da), oligopeptides, oligonucleotides, or carbohydrates capable of activating or inhibiting the SKN-1-mediated oxidative stress response.

The screening methods are useful, for example, for isolating novel antioxidants (e.g., compounds that activate a stress response pathway, e.g., an oxidative stress response pathway, e.g., the SKN-1-mediated oxidative stress response pathway) or compounds that can be used as pesticides (e.g., compounds that inhibit a stress response pathway, e.g., an oxidative stress response pathway, e.g., the SKN-1-mediated oxidative stress response pathway). Skilled practitioners will also appreciate that the screening methods described herein can be used, for example, to identify or isolate other genes and/or proteins involved in a stress response pathway, e.g., an oxidative stress pathway, e.g., the SKN-1-mediated oxidative stress response pathway, which can themselves be used as pharmaceutical agents or as potential targets for drug discovery.

In Vivo Screening Methods

In one embodiment, the present invention provides methods for determining whether a test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound. One method includes providing a nematode, isolated nematode cell, or isolated mammalian cell capable of expressing SKN-1 and containing at least one (i.e., one or more) transgene comprising an oxidative stress resistance gene promoter operably linked to a reporter gene; and contacting the nematode, isolated nematode cell, or isolated mammalian cell, with the test compound, wherein an increase in expression of the transgene following contact of the nematode, isolated nematode cell, or isolated mammalian cell, with the test compound indicates that the test compound is a candidate stress response-activating

compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

A similar method can be carried out to determine whether a test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound. The method includes providing a nematode, isolated nematode cell, or isolated mammalian cell capable of expressing SKN-1 and containing at least one (i.e., one or more) transgene comprising an oxidative stress resistance gene promoter operably linked to a reporter gene; contacting the nematode, isolated nematode cell, or isolated mammalian cell, with the test compound; and subjecting the nematode, isolated nematode cell, or isolated mammalian cell to conditions that activate the SKN-1-mediated oxidative stress response in the absence of the test compound, wherein a decrease or lack of increase in expression of the transgene following the subjecting step indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

In still another method, a nematode, isolated nematode cell, or isolated mammalian cell capable of expressing a SKN-1 fusion protein is utilized to determine whether a test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound. The method includes providing a nematode, isolated nematode cell, or isolated mammalian cell containing a transgene that includes SKN-1 DNA operably linked to a reporter gene; contacting the nematode, isolated nematode cell, or isolated mammalian cell, with the test compound; and determining whether the SKN-1 fusion protein accumulates in nuclei in the nematode, isolated nematode cell, or isolated mammalian cell. Increased accumulation (e.g., above control levels), indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

A similar method can be carried out to determine whether a test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound. The method includes providing a nematode, isolated nematode cell, or isolated mammalian cell containing a transgene that includes SKN-1 DNA operably linked to a reporter gene; contacting the nematode, isolated nematode cell, or isolated mammalian cell, with the test compound; subjecting the nematode, isolated nematode cell, or isolated mammalian cell to conditions that activate the SKN-1-mediated oxidative stress response in the absence of the test compound; and determining whether the SKN-1 fusion protein accumulates in nuclei in the nematode, isolated nematode cell, or isolated mammalian cell. Decreased or no increase in accumulation (e.g., as compared to control levels), indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

A "test compound" can be any chemical compound, for example, a macromolecule (e.g., a polypeptide, a protein complex, or a nucleic acid) or a small molecule (e.g., an amino acid, a nucleotide, an organic or inorganic compound). The test compound can have a formula weight of less than about 10,000 grams per mole, less than 5,000 grams per mole, less than 1,000 grams per mole, or less than about 500 grams per mole. The test compound can be naturally occurring (e.g., an herb or a natural product), synthetic, or can include both natural and synthetic components. Examples of small molecules include peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, and organic or inorganic compounds, e.g., heteroorganic or organometallic compounds.

Test compounds can be screened individually or in parallel. An example of parallel screening is a high throughput drug screen of large libraries of chemicals. Such libraries of test compounds can be generated or purchased, e.g., from Chembridge Corp., San Diego, CA. Libraries can be designed to cover a diverse range of compounds. For example, a library can include 500, 1000, 10,000, 50,000, or 100,000 or more unique compounds. Alternatively, prior experimentation and

anecdotal evidence can suggest a class or category of compounds of enhanced potential. A library can be designed and synthesized to cover such a class of chemicals.

Examples of methods for the synthesis of molecular libraries can be found in, for example, DeWitt et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90:6909; Erb et al. (1994) Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al. (1994). J. Med. Chem. 37:2678; Cho et al. (1993) Science 261:1303; Carrell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2059; Carell et al. (1994) Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al. (1994) J. Med. Chem. 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382; Felici (1991) *J. Mol. Biol.* 222:301-310; Ladner *supra.*).

If a nematode is to be used in the present methods, it can be of any genus and species, e.g., any animal, plant, or insect parasitic nematode, or any free-living terrestrial or aquatic (i.e., marine or freshwater) nematode, that is capable of harboring one or more of the constructs described herein. An example of a particularly useful nematode is *C. elegans*. Likewise, if a cultured nematode cell (e.g., a cultured nematode intestinal cell) is to be used, it can be isolated from any genus and species of nematode. Further, if a mammalian cell is to be used, it can be any mammalian cell, e.g., 3T3, HeLa, and/or HD3 erythroblasts, among others.

The oxidative stress resistance gene promoter can be from a gene described herein as, or identified using a method described herein, to be a target of a SKN-1 polypeptide. As used herein, a "promoter" is a minimal sequence sufficient to direct transcription located in the 5' region of the native gene. For example, the promoter can be from a gene encoding γ-glutamine cysteine synthase heavy chain (GCS(h)) (*gcs-1*, sequences for which are set forth in WormBase as F37B12.2); glutathione synthetase (sequences for which are set forth in WormBase as M176.2); NADH quinone oxidoreductase (set forth in WormBase as F39B2.3); superoxide dismutase

(*sod-1*, *sod-2*, and *sod-3*; sequences for which are set forth in WormBase as C15F1.7; F10D11.1; C08A9.1, respectively); catalase (*ctl-1*; sequences for which are set forth in WormBase as Y54G11A.6); and several forms of glutathione S-transferase (sequences for which are set forth in WormBase as R03D7.6, F35E8.8, F11G11.2, and K08F4.7), among others. In certain embodiments of the present invention, a SKN-1 promoter is included in a transgene (sequences for which are set forth in the WormBase database as T19E7.2a, T19E7.2b, and T19E7.2c).

The present invention contemplates that promoters can be modified to provide preferential (i.e., organ- and/or tissue-specific) expression (and/or repression) of a construct. Examples of such modifications are described in detail in Example 1 (below). There, *gcs-1* promoter deletions were constructed using PCR. Predicted SKN-1 polypeptide binding sites (underlined) were altered as follows: Site 1 –608 GATGACAAT to CTGCAGAAT, Site 2 –317 GATGACTTA to CTGCAGTTA, and Site 3 –121 TTTATCATC to TTTCTGCAG. Expression patterns of the construct changed depending upon the deletions made. For example, the *gcsΔ 2* mutation eliminated pharyngeal GCS-1::GFP expression, but allowed near-wild type levels of ASI and intestinal expression. Concurrent ablation of SKN-1 binding site 3 (*gcsΔ 2,mut3*) eliminated transgene expression in all tissues. Skilled practitioners will appreciate that similar procedures could be used to cause preferential expression of other constructs.

The "reporter gene" can be any sequence the expression of which can be detected or measured, other than the coding sequence to which the promoter naturally is operably linked. Typically, the reporter gene is heterologous to the nematode, isolated nematode cell, or isolated mammalian cell in which promoter activity is measured. Examples of reporter genes include, without limitation, genes that encode green fluorescent protein (or any other fluorescent marker), chloramphenicol acetyl transferase (*cat*), ß-glucuronidase (*gus*), ß-Galactosidase (*lacZ*), luciferase, and the like. Reporter gene expression can be measured by any of a number of conventional methods, and the optimal method will depend upon factors such as the nature and function of the reporter gene. In general, suitable assays of reporter gene expression include methods such as (i) assaying the function of a product of the reporter gene (e.g., measuring an enzymatic reaction catalyzed by a product of the reporter gene);

(ii) measuring the level of protein expressed from the reporter gene (e.g., by SDS-PAGE or in an immunoassay using antibodies (e.g., polyclonal or monoclonal antibodies) that specifically bind to the product of the reporter gene); and (iii) measuring the level of mRNA transcribed from the reporter gene. Included within the invention are assays that permit high throughput screening of test compounds.

Reporter genes, and methods for measuring their expression, are well known to those of ordinary skill in the art. Methods for making the transgenes and their incorporation into the genomes of organisms, e.g., nematodes, or cells, e.g., cultured nematode or mammalian cells, are also well known in the art. Example 1 (below) describes in detail how to make *gcs-1::gfp* and *skn-1::gfp* transgenes, how to incorporate such transgenes into the genome of *C. elegans*, and how to measure expression of the transgenes (a microscopy-based screen is performed in that Example). Skilled practitioners will appreciate that similar protocols can be used to create other transgenes described herein, and to incorporate those transgenes into other types of nematodes and cultured cells. The present invention contemplates that more than one type of transgene can be inserted into the genome of a nematode or cell.

Compounds capable of activating a stress response, e.g., an oxidative stress response, e.g., the SKN-1-mediated oxidative stress response, in an initial screen discussed above can be considered candidate stress response-activating compounds, e.g., candidate oxidative stress response-activating compounds, e.g., candidate SKN-1-mediated oxidative stress response-activating compounds. Such candidate compounds can be subjected to a confirmatory step, e.g., to determine whether the candidate compound increases the overall oxidative stress resistance of a nematode or cultured cell (e.g., a mammalian cell). Methods for evaluating the oxidative stress resistance of a cell or organism (e.g., relative to controls) are well known in the art, and include, for example, subjecting an organism or cell to conditions of oxidative stress (e.g., using known compounds or by increasing the temperature of the culture environment) and measuring overall survival rate. Alternatively or in addition, the confirmatory step can involve determining whether the candidate compound is capable of increasing expression of at least one gene involved in the vertebrate Nrf oxidative stress response pathway, using transgenes (similar to those described herein)

or any other method known in the art for measuring increased expression (e.g., Western blotting). Candidate activating compounds that increase stress resistance, e.g., oxidative stress resistance, e.g., SKN-1-mediated oxidative stress resistance, in a nematode, cultured nematode cell, or mammalian cell, or that are capable of increasing expression of at least one gene involved in the vertebrate Nrf oxidative stress response pathway, can be considered stress response-activating agents, e.g., oxidative stress response-activating agents, e.g., SKN-1-mediated oxidative stress response-activating agents.

Alternatively or in addition, a different confirmatory step can be carried out. This step involves providing a nematode not capable of expressing a SKN-1 polypeptide (i.e., a *skn-1* mutant) containing at least one transgene that includes an oxidative stress resistance gene promoter operably linked to a reporter gene; and contacting the nematode with the test compound. If the transgene displays no increase in expression, the candidate compound specifically activates the SKN-1-mediated oxidative stress response, and can be considered an oxidative stress response-activating agent.

Compounds capable of inhibiting a stress response, e.g., an oxidative stress response, e.g., the SKN-1-mediated oxidative stress response, in an initial screen discussed above can be considered candidate inhibiting compounds, i.e., candidate stress response-inhibiting compounds, e.g., candidate oxidative stress response-inhibiting compounds, e.g., candidate SKN-1-mediated oxidative stress response-inhibiting compounds. Such candidate inhibitory compounds can also be subjected to a confirmatory step, e.g., to determine whether the candidate inhibitory compound decreases the overall oxidative stress resistance of a nematode or cultured cell (e.g., a mammalian cell). Alternatively or in addition, the confirmatory step can involve determining whether the candidate compound is capable of decreasing (or preventing increased expression under conditions of oxidative stress) expression of at least one gene involved in the vertebrate Nrf oxidative stress response pathway (or an oxidative stress resistance gene not utilized in the initial screen). Such screens can be carried out using transgenes similar to those described herein or any other method known in the art for measuring increased or decreased expression (e.g., Western blotting). Candidate inhibiting compounds that decrease oxidative stress resistance in a

nematode, cultured nematode cell, or mammalian cell, or prevent (or decrease) increased expression of another oxidative stress resistance gene under conditions of oxidative stress, can be considered an oxidative stress response-inhibiting agent.

Some screens of the present invention require subjecting the nematode or cell to conditions that activate the SKN-1-mediated oxidative stress response in the absence of a test compound. Such conditions include exposing the nematode or cell to a known antioxidant, e.g., sulforaphane. Other conditions include, e.g., exposure to the herbicide paraquat (methyl viologen), heat, $CdCl_2$, arsenite, H_2O_2, diamide, and/or sodium azide.

In Vitro Screening Methods

The invention also provides *in vitro* methods for determining whether a test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound. For example, the method can include contacting a SKN-1 polypeptide, or a fragment thereof (e.g., a SKN-1 Domain), a SKN-1 DNA, a GSK-3 polypeptide, or a fragment thereof, or a GSK-3 DNA with a test compound; and detecting interaction of the test compound with the SKN-1 or GSK-3 polypeptide (or fragment thereof) or with the SKN-1 or GSK-3 DNA. An interaction (e.g., direct or indirect binding) with SKN-1 polypeptide or DNA indicates that the test compound is a candidate stress response-inhibiting compound, e.g., a candidate oxidative stress response-inhibiting compound, e.g., a candidate SKN-1-mediated oxidative stress response-inhibiting compound. An interaction (e.g., direct or indirect binding) with GSK-3 polypeptide or DNA indicates that the test compound is a candidate stress response-activating compound, e.g., a candidate oxidative stress response-activating compound, e.g., a candidate SKN-1-mediated oxidative stress response-activating compound.

Another method takes advantage of interactions between SKN-1 polypeptides and oxidative stress resistance genes that are targets of SKN-1 polypeptides. The method includes providing a polypeptide that includes a SKN-1 polypeptide (or fragment thereof that is capable of interacting (i.e., binding) with an oxidative stress resistance gene, e.g., a SKN-1 Domain), and an oxidative stress resistance gene. The

oxidative stress resistance gene can be a gene identified herein as being a target for SKN-1 polypeptides, e.g., genes encoding γ-glutamine cysteine synthase heavy chain, glutathione synthetase, NADH quinone oxidoreductase, superoxide dismutase, catalase, or glutathione S-transferase. Alternatively, a nucleic acid sequence that includes a SKN-1 polypeptide-binding fragment of the oxidative stress resistance gene can be provided. Examples of SKN-1 polypeptide binding fragments for a number of oxidative stress resistance genes are provided in Table 1. The method further includes contacting the polypeptide comprising the SKN-1 polypeptide (or fragment thereof) and a nucleotide sequence comprising an oxidative stress resistance gene (or SKN-1 polypeptide-binding fragment thereof) with a test compound; and determining whether the SKN-1 polypeptide (or fragment thereof) and the oxidative stress resistance gene (or SKN-1 polypeptide-binding fragment thereof) interact (i.e., bind) in the presence of the test compound. If no or decreased interaction is evident, the test compound can be considered a candidate SKN-1-mediated oxidative stress response-inhibiting compound.

As in the in vivo screening methods, compounds isolated using the in vitro methods can be subjected to any confirmatory step herein described herein. Skilled practitioners will appreciate that in any screening method described herein, homologues of SKN-1 or GSK-3 (e.g., genes or polypeptides) can be substituted for the SKN-1 or GSK-3 DNA or polypeptides. Where such substitutions are made, the screens can be carried out essentially as described herein.

Medicinal Chemistry

Once a compound (or agent) of interest has been identified, standard principles of medicinal chemistry can be used to produce derivatives of the compound. Derivatives can be screened for improved pharmacological properties, for example, efficacy, pharmaco-kinetics, stability, solubility, and clearance. The moieties responsible for a compound's activity in the assays described above can be delineated by examination of structure-activity relationships (SAR) as is commonly practiced in the art. A person of ordinary skill in pharmaceutical chemistry can modify moieties on a lead compound and measure the effects of the modification on the efficacy of the compound to thereby produce derivatives with increased potency. For an example,

see Nagarajan *et al.* (1988) *J. Antibiot.* 41: 1430-8. Furthermore, if the biochemical target of the compound (or agent) is known or determined, the structure of the target and the compound can inform the design and optimization of derivatives. Molecular modeling software is commercially available (e.g., Molecular Simulations, Inc.) for this purpose.

Isolated Compounds

Skilled practitioners will appreciate that compounds isolated using the screens described herein can be used to treat a number of conditions or disorders.

Oxidative stress contributes to human pathologies that include diabetes, atherosclerosis, neurodegenerative diseases, reperfusion injury, and HIV infection (see, e.g., Finkel et al., Nature 408: 239-247 (2000). The ROS defenses mobilized by human Nrf proteins are thought to be beneficial in these diverse disease states. This gene activation pathway is also important for drug detoxification, and, therefore, for chemotherapeutic agent tolerance, and it may provide a widely applicable means of cancer prevention (see, e.g., Chan et al. Proc Natl Acad Sci U.S.A 98: 4611-6. (2001); Hayes et al., Cancer Lett 174: 103-113 (2001); and Wolf *Proc Natl Acad Sci* U.S.A 98: 2941-2943 (2001)). For example, dietary consumption of chemoprotective antioxidants acts through Nrf2 to inhibit chemical carcinogenesis in mice, and decreases the risk of gastrointestinal and lung tumors in humans (see, e.g., Ramos-Gomez et al. Proc Natl Acad Sci U.S.A 98: 3410-3415 (2001); Fahey et al. Proc Natl Acad Sci U.S.A 99: 7610-7615 (2002); and Thimmulappa et al. Cancer Res 62: 5196-5203 (2002)).

Accordingly, candidate stress response-activating compounds, e.g., candidate oxidative response-activating compounds, e.g., candidate SKN-1-mediated oxidative stress response-activating compounds, shown to increase the oxidative stress resistance of mammalian cells (oxidative stress response-activating agents) can be used as novel antioxidants. Such antioxidants could be used to treat a number of conditions including, but not limited to, aging, cancer (e.g., wherein such an agent can be used in chemoprevention), arteriosclerosis, the effects of diabetes (e.g., the neuropathy and vascular complications associated therewith, islet cell destruction, and detrimental insulin responses), neurodegenerative diseases (e.g., by increasing

neuronal oxidative stress resistance and, therefore, survival), reperfusion injury (e.g., injury arising from oxidative stress caused by hypotension, myocardial infarction, and/or stroke); the effects of sleep apnea (e.g., vascular injury arising from the cycle of hypoxia/reoxygenation); viral infection (e.g., human immunodeficiency virus infection); bacterial infections (e.g., in the gut); and toxicity (e.g., drug toxicity (e.g., arising from chemotherapy), heavy metal toxicity, and hepatic toxicity).

Inhibitors are also useful in certain clinical settings. For example, candidate stress response-inhibiting compounds, e.g., candidate oxidative stress response-inhibiting compounds, e.g., candidate SKN-1-mediated oxidative stress response-inhibiting compounds, shown to reduce the oxidative stress resistance of mammalian cells (oxidative stress response-inhibiting agents) can be used to eliminate undesirable cells in an animal, e.g., tumor cells in a human. Further, in certain cancer cells, the oxidative stress response pathway is up-regulated, conferring growth advantages. An inhibitor can be used as a component of a chemotherapeutic regimen to prevent such up-regulation. As still another example, reactive oxygen species (ROS) production plays a detrimental role in certain inflammatory responses, which can be prevented or decreased using an inhibitor described herein.

Modulation of the SKN-1-Mediated Oxidative Stress Response

The invention provides methods for modulating the SKN-1-mediated oxidative stress response that fall into two basic categories: inhibiting (e.g., limiting or reducing) a stress response, e.g., an oxidative stress response, e.g., the SKN-1-mediated oxidative stress response, and activating, e.g., supplementing or providing oxidative stress response activity where there is insufficient or no activity. Whether the SKN-1 mediated oxidative stress response is inhibited or increased depends on the intended application.

Inhibition of the Response by SKN-1 Inhibitors

In some embodiments, the invention provides for inhibiting the SKN-1-mediated oxidative stress response. Agents that inhibit can be used as, e.g., novel pesticides to control insects or nematodes (e.g., pathogenic nematodes). Agents that inhibit the SKN-1-mediated oxidative stress response are useful to inhibit nematode

reproduction, decrease nematode lifespan, and increase nematode sensitivity to oxidative stresses (thereby making the nematode easier to eradicate, e.g., using known pesticides). Such agents are also useful for inhibiting SKN-1 activation of the oxidative stress response in a laboratory/research setting in order to identify other genes and/or proteins involved in this oxidative stress response pathway.

For example, an antisense nucleic acid effective to inhibit expression of an endogenous SKN-1 gene can be utilized. The antisense nucleic acid can include a nucleotide sequence complementary to an entire SKN-1 RNA or only a portion of the RNA. On one hand, the antisense nucleic acid needs to be long enough to hybridize effectively with the SKN-1 RNA. Therefore, the minimum length is approximately 10, 11, 12, 13, 14, or 15 nucleotides. On the other hand, as length increases beyond about 150 nucleotides, effectiveness at inhibiting translation increases only marginally, while difficulty in introducing the antisense nucleic acid into target cells may increase significantly. In view of these considerations, a preferred length for the antisense nucleic acid is from about 15 to about 150 nucleotides, e.g., 20, 25, 30, 35, 40, 45, 50, 60, 70, or 80 nucleotides. The antisense nucleic acid can be complementary to a coding region of SKN-1 mRNA or a 5' or 3' non-coding region of a SKN-1 mRNA (or both). One approach is to design the antisense nucleic acid to be complementary to a region on both sides of the translation start site of the SKN-1 mRNA.

The antisense nucleic acid can be chemically synthesized, e.g., using a commercial nucleic acid synthesizer according to the vendor's instructions. Alternatively, the antisense nucleic acids can be produced using recombinant DNA techniques. An antisense nucleic acid can incorporate only naturally occurring nucleotides. Alternatively, it can incorporate variously modified nucleotides or nucleotide analogs to increase its in vivo half-life or to increase the stability of the duplex formed between the antisense molecule and its target RNA. Examples of nucleotide analogs include phosphorothioate derivatives and acridine-substituted nucleotides. Given the description of the targets and sequences, the design and production of suitable antisense molecules is within ordinary skill in the art. For guidance concerning antisense nucleic acids, see, e.g., Goodchild, "Inhibition of Gene

Expression by Oligonucleotides," in *Topics in Molecular and Structural Biology, Vol. 12: Oligodeoxynucleotides* (Cohen, ed.), MacMillan Press, London, pp. 53-77.

Delivery of antisense oligonucleotides can be accomplished by any method known to those of skill in the art. For example, delivery of antisense oligonucleotides for cell culture and/or ex vivo work can be performed by standard methods such as the liposome method or simply by addition of membrane-permeable oligonucleotides. To resist nuclease degradation, chemical modifications such as phosphorothionate backbones can be incorporated into the molecule.

Delivery of antisense oligonucleotides for *in vivo* applications can be accomplished, for example, via local injection of the antisense oligonucleotides at a selected site. This method has previously been demonstrated for psoriasis growth inhibition and for cytomegalovirus inhibition. *See*, for example, Wraight et al., (2001). *Pharmacol Ther*. Apr; 90(1):89-104.; Anderson, et al., (1996) *Antimicrob Agents Chemother* 40: 2004-2011; and Crooke et al., *J Pharmacol Exp Ther* 277: 923-937.

Similarly, the present invention anticipates that RNA interference (RNAi) techniques could be used to inhibit the SKN-1-mediated oxidative stress response, in addition or as an alternative to, the use of antisense techniques. For example, small interfering RNA (siRNA) duplexes directed against SKN-1, or any oxidative stress response gene target of SKN-1, could be synthesized and used to prevent expression of the encoded protein(s). Skilled practitioners will also appreciate that RNAi techniques could be used in screens to identify other genes and/or proteins that modulate the SKN-1 oxidative stress response pathway. For example, these techniques could be used in a screen for genes that when inhibited allowed constitutive activation of the *gcs-1::gfp* transgene, or that prevented the *gcs-1::gfp* transgene from being activated by oxidative stress or antioxidants.

As another example, SKN-1 polypeptide activity can be inhibited using a SKN-1 polypeptide binding molecule such as an antibody, e.g., an anti-SKN-1 polypeptide antibody, or a SKN-1 polypeptide -binding fragment thereof. The anti-SKN-1 polypeptide antibody can be a polyclonal or a monoclonal antibody. Alternatively or in addition, the antibody can be produced recombinantly, e.g., produced by phage display or by combinatorial methods as described in, e.g., Ladner

et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

As used herein, the term "antibody" refers to a protein comprising at least one, and preferably two, heavy (H) chain variable regions (abbreviated herein as VH), and at least one and preferably two light (L) chain variable regions (abbreviated herein as VL). The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) *J. Mol. Biol.* 196:901-917). Each VH and VL is composed of three CDR's and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

An anti-SKN-1 polypeptide antibody can further include a heavy and light chain constant region, to thereby form a heavy and light immunoglobulin chain, respectively. The antibody can be a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region is comprised of three domains, CH1, CH2, and CH3. The light chain constant region is comprised of one domain, CL. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the

antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (Clq) of the classical complement system.

A "SKN-1 polypeptide-binding fragment" of an antibody refers to one or more fragments of a full-length antibody that retain the ability to specifically bind to SKN-1 polypeptide or a portion thereof. "Specifically binds" means that an antibody or ligand binds to a particular target to the substantial exclusion of other substances. Examples of SKN-1 polypeptide binding fragments of an anti-SKN-1 polypeptide antibody include, but are not limited to: (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are encoded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain antibodies are also encompassed within the term "SKN-1 polypeptide-binding fragment" of an antibody. These antibody fragments can be obtained using conventional techniques known to those with skill in the art.

The anti-SKN-1 polypeptide antibody can be a fully human antibody (e.g., an antibody made in a mouse which has been genetically engineered to produce an antibody from a human immunoglobulin sequence), or a non-human antibody, e.g., a rodent (mouse or rat), goat, primate (e.g., monkey), camel, donkey, porcine, or fowl antibody.

An anti-SKN-1 polypeptide antibody can be one in which the variable region, or a portion thereof, e.g., the CDRs, are generated in a non-human organism, e.g., a rat or mouse. The anti-SKN-1 polypeptide antibody can also be, for example, chimeric, CDR-grafted, or humanized antibodies. The anti-SKN-1 polypeptide

antibody can also be generated in a non-human organism, e.g., a rat or mouse, and then modified, e.g., in the variable framework or constant region, to decrease antigenicity in a human.

Another approach to inhibiting SKN-1 activity is the administration of a SKN-1 antagonist that binds to (i.e., blocks) SKN-1 polypeptides and prevents it from interacting with its target gene (e.g., a gene involved in the oxidative stress response, e.g.,. a Phase II detoxification gene). Such SKN-1 polypeptide antagonists can be identified using a screening method described herein. Alternatively, the SKN-1 antagonist can be an anti-SKN-1 polypeptide antibody, or fragment thereof, as described above.

Inhibition of the Response by GSK-3 Activators

A stress response, e.g., an oxidative stress response, e.g., an SKN-1-mediated oxidative response, can be inhibited by supplementing or providing new GSK-3 activity. For example, new or supplemental GSK-3 activity can be provided in vivo by direct administration of a recombinant GSK-3 polypeptide, e.g., to pathogenic nematodes prior to, during, and/or after their introduction into the environment of interest. GSK-3 polypeptides that can be used to supplemental GSK-3 activity are described herein. Such polypeptides can be used "as is" or modified. Examples of modifications include derivation of amino acid side chains, glycosylation, conservative amino acid substitutions, and chemical conjugation or fusion to other non-GSK-3 polypeptide moieties.

Alternatively or in addition, a GSK-3 polypeptide can be introduced indirectly into an organism, e.g., a nematode, by expressing within the cells of the organism a nucleic acid construct containing a nucleotide sequence encoding a GSK-3 polypeptide. Any appropriate expression vector suitable for transfecting the cells of the organism of interest can be used in the invention. The nucleic acid construct can be derived from a non-replicating linear or circular DNA or RNA vector, or from an autonomously replicating plasmid or viral vector. Methods for constructing suitable expression vectors are known in the art, and useful materials are commercially available.

Activation of the Response by SKN-1 Activators

In some embodiments, the invention provides for activating the SKN-1-mediated oxidative stress response. Agents that activate can be used, e.g., to increase the oxidative stress resistance of beneficial pathogenic nematodes, e.g., those used to protect crops by eliminating/controlling the population of certain insects. Examples of such nematodes are *Steinernema carpocapsae*, *S. Glaseri* and *Heterorhabditis* spp. Such agents are also useful for, e.g., activating the oxidative stress response via SKN-1 in a laboratory/research setting in order to identify other genes and/or proteins involved in the oxidative stress response pathway.

For example, new or supplemental SKN-1 activity can be provided in vivo by direct administration of a recombinant SKN-1 polypeptide, e.g., to pathogenic nematodes prior to, during, and/or after their introduction into the environment of interest. SKN-1 polypeptides that can be used to supplemental SKN-1 activity are described herein, e.g., SEQ ID NO:2, or a fragment thereof. Another is described in Example 1. There, a SKN-1/green fluorescent protein fusion protein is described. Such polypeptides can be used "as is" or modified. Examples of modifications include derivation of amino acid side chains, glycosylation, conservative amino acid substitutions, and chemical conjugation or fusion to other non-SKN-1 polypeptide moieties.

Alternatively or in addition, a SKN-1 polypeptide can be introduced indirectly into an organism, e.g., a nematode, by expressing within the cells of the organism a nucleic acid construct containing a nucleotide sequence encoding a SKN-1 polypeptide. Any appropriate expression vector suitable for transfecting the cells of the organism of interest can be used in the invention. The nucleic acid construct can be derived from a non-replicating linear or circular DNA or RNA vector, or from an autonomously replicating plasmid or viral vector. Methods for constructing suitable expression vectors are known in the art, and useful materials are commercially available. With respect to nematodes, an example for producing transgenic *skn-1::gfp* strains of *C. elegans* is discussed in detail in Example 1.

Another approach to increasing SKN-1 activity is the administration of an antioxidant (e.g., sulforaphane) or other compound, e.g., a compound isolated using

one of the screening methods described above. Such a compound can be, e.g., a small organic or inorganic molecule, e.g., a novel antioxidant.

Activation of the Response by GSK-3 Inhibitors

A stress response, e.g., an oxidative stress response, e.g., an SKN-1-mediated oxidative response, can be activated by an inhibitor of GSK-3 expression or activity. Any of the methods described for inhibiting SKN-1 expression or activity described herein, e.g., the use of antisense or antibodies, can be used to inhibit the expression or activity of GSK-3. GSK-3 activity can also be inhibited by administering a GSK-3 antagonist that binds to (i.e., blocks) GSK-3 polypeptides. GSK-3 antagonists are known, and include, e.g., lithium, SB-415286 (see, e.g., MacAulay et al. (2003) *Eur. J. Biochem.*, 270:3829-38), GBP (see, e.g., Yost et al. (1998) *Cell*, 93:1031-41), and AR-A014418 (see, e.g., Bhat et al. (2003) *J. Biol. Chem.*, 278:45937-45).

Pharmaceutical Compositions

The compounds, nucleic acids, and polypeptides, fragments thereof, as well as antibodies, e.g., anti-SKN-1 polypeptide antibodies, anti-GSK-3 polypeptide antibodies, other molecules and agents of the invention (also referred to herein as "active compounds"), e.g., novel antioxidants, can be incorporated into pharmaceutical compositions. Such compositions typically include the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. A "pharmaceutically acceptable carrier" can include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic

solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be achieved by including an agent that delays absorption, e.g., aluminum monostearate and gelatin in the composition.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other

ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of, e.g., tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use, e.g., as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose; a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue, e.g., bone or cartilage, in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the

dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

For the SKN-1 or GSK modulating agents described herein, an effective amount, e.g., of a protein or polypeptide (i.e., an effective dosage), ranges from about 0.001 to 30 mg/kg body weight, e.g. about 0.01 to 25 mg/kg body weight, e.g. about 0.1 to 20 mg/kg body weight. The protein or polypeptide can be administered one time per week for between about 1 to 10 weeks, e.g. between 2 to 8 weeks, about 3 to 7 weeks, or for about 4, 5, or 6 weeks. The skilled artisan will appreciate that certain factors influence the dosage and timing required to effectively treat a patient, including but not limited to the type of patient to be treated, the severity of the disease or disorder, previous treatments, the general health and/or age of the patient, and other diseases present. Moreover, treatment of a patient with a therapeutically effective amount of a protein, polypeptide, antibody, or other compound can include a single treatment or, preferably, can include a series of treatments.

For antibodies, a useful dosage is 0.1 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg). Generally, partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly, lower dosages and less frequent administration are possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration. A method for lipidation of antibodies is described by Cruikshank *et al.* ((1997) *J. Acquired Immune Deficiency Syndromes and Human Retrovirology* 14:193).

If the agent is a small molecule, exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1

microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (e.g., a human) to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

Nucleic acid molecules (e.g., SKN-1 or GSK-3 DNA) of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see, e.g., U.S. Patent 5,328,470) or by stereotactic injection (see, e.g., Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

The invention is illustrated in part by the following examples, which are not to be taken as limiting the invention in any way.

Example 1: SKN-1 Links *C. elegans* Mesendodermal Specification to a Conserved Oxidative Stress Response

***C. elegans* strains and bioinfomatics**

Strains were maintained at 20°C unless otherwise noted, using standard methods (Brenner, Genetics 77: 71-94 (1974)). Alleles used were N2 Bristol as the wild-type, and *skn-1(zu67)* and (*zu129*) (Bowerman et al. Cell 68: 1061-1075 (1992)). *C. elegans* orthologs of Nrf targets and other detoxification genes were identified by searching WORMpep or genomic databases (Sanger Centre). Predicted SKN-1 sites (Fig. 1C) 5' of their coding regions were identified with TFSEARCH (Heinemeyer et al. Nucleic Acids Res 26: 362-367 (1998)).

Paraquat sensitivity and lifespan assays

To assay sensitivity to paraquat, young adults were transferred from NGM agar plates into 24-well plates (6 per well) containing 0.3 µl of M9 that either did or did not contain 100mM paraquat. Worms were incubated at 20°C, and the number of dead animals was counted by the continuous absence of swimming movements and pharyngeal pumping. Lifespan assays were performed essentially as described by Hsin et al. (Nature 399: 362-366 (1999)). Animals were transferred to new plates daily and classified as dead when they did not move after repeated prodding with a pick. Animals that crawled away from the plate, exploded, or contained internally hatched worms were excluded from the analysis.

Plasmid constructions

All PCR was performed using Pfu polymerase (Stratagene). GFP vectors pPD95.67 and pPD114.35 were obtained. A *skn-1::gfp* promoter fusion construct (SknPro::GFP; Fig. 5A) was created by ligating GFP vector pPD95.67 and an PCR-amplified 2.1 kb clone containing the promoter region and 38 amino acids from first ATG codon of the *skn-1* gene from cosmid T19E7 (sequence information about this cosmid can be accessed in WormBase under number T19E7). To generate the SKN-1::GFP translational fusion construct (Fig. 5A), the 5.7 kb EcoRI DNA fragment that rescues the maternal *skn-1* phenotype and encodes the 533 amino acid SKN-1 protein (Bowerman et al. *Cell* 68: 1061-1075 (1992)) was amplified from cosmid B0547. A

ClaI site was created immediately 3' to the SKN-1 C-terminus by the Quick Change method (Stratagene), which was used for all site-directed mutagenesis. This EcoRI fragment was subcloned into pUC18 that contained the upstream 1.3 kb SphI-EcoRI fragment from SknPro::GFP (Fig. 5A). A 0.8 kb ClaI fragment that contained the GFP open reading frame (amplified from plasmid pPD114.35) was then cloned into the ClaI site to generate an in-frame exon fusion of GFP to the SKN-1 C-terminus.

The *C. elegans gcs-1* ORF (WormBase number F37B12.2) is between 45% and 54% identical to human, mouse, *Drosophila* and yeast GCS(h) (data not shown). To construct the *gcs-1::gfp* transgene, a fragment that contained 1840 bp upstream of the initiation ATG, along with sequences encoding the 17 amino terminal GCS-1 residues, was amplified by PCR from cosmid F37B12, and cloned into GFP vector pPD95.67. Promoter deletions were similarly constructed by PCR. In *gcs-1* point mutation constructs, predicted SKN-1 sites (underlined) were altered as follows: Site 1 –608 GATGACAAT to CTGCAGAAT, Site 2 –317 GATGACTTA to CTGCAGTTA, and Site 3 –121 TTTATCATC to TTTCTGCAG.

Transgenic analyses

Transgenic strains were generated by injecting DNA into the gonad of young adult animals as described in Mello et al. (EMBO Journal **10**: 3959-3970 (1991)). *gcs-1::gfp* transgene constructs (Fig. 3A) were injected at 50 ng/µl along with the *rol-6* marker (pRF4) at 100 ng/µl. Between three and six independent extrachromosomal lines were generated and analyzed for each *gcs-1::gfp* construct. To investigate GCS-1::GFP expression in the *skn-1(zu67)* background, *rol-6*–marked *gcs-1::gfp* hermaphrodites were mated with N2 males, then their transgenic progeny were crossed with *skn-1(zu67)/DnT1* hermaphrodites, which have an *unc* phenotype. After transgenic males were successively crossed twice with *skn-1(zu67)/DnT1* hermaphrodites, *unc; rol* F3 hermaphrodite progeny were selected. From this population, *skn-1(zu67)/DnT1; gcs-1::gfp* animals were identified on the basis of their non-*unc; rol* progeny laying dead eggs. Two different *gcs-1::gfp* lines were thereby crossed into the *skn-1(zu67)* background and examined for GFP expression. DIC and fluorescence images were acquired with a Zeiss AxioSKOP2 microscope and AxioCam cooled color digital camera.

To investigate expression of *gcs-1::gfp* and mutant transgenes, worms were exposed to oxidative stress under the following conditions. For heat shock, worms cultured at 20°C were transferred onto prewarmed seeded plates and incubated at 29°C for 20 hours, then examined by fluorescence microscopy for GFP expression. *gcs-1::gfp* induction was also observed in an alternative heat treatment protocol, during which worms cultured at 20°C were transferred onto prewarmed plates and incubated at 34°C for 2 to 4 hours, then returned to 20°C and examined for GFP expression hourly during a four hour recovery period. In the experiments described in Table 2, young adults were transferred to plates that contained 1mM paraquat in the agar and maintained at 20°C for 3 days prior to analysis. In an alternative induction protocol, worms that carried *gcs-1::gfp* or the mutant transgenes shown in Figure 3A were incubated in M9 either with or without 100 mM paraquat for 30 minutes, then allowed to recover on plates for four hours. The latter procedure also resulted in induction of intestinal *gcs-1::gfp* expression by paraquat but was associated with a higher background in uninduced animals.

To create transgenic *skn-1::gfp* strains, 2.5, 10, or 50 ng/µl of transgene DNA (Fig. 5A) was injected into N2 animals at along with 100 ng/µl of pRF4 to generate extrachromosomal transgenic lines. Two different extrachromosomal arrays, *Ex001* and *Ex007*, generated with 2.5 and 10 ng/µl of SKN-1::GFP, respectively, were integrated into the chromosome by UV irradiation (400J/m2) to produce the insertion strains *Is001* and *Is007*, respectively. To rescue the embryonic lethality of a *skn-1* mutation, SKN-1::GFP was injected into *skn-1(zu67)/DnT1* animals at 2.5 ng/µl with 100 ng/µl of the pRF4 marker. Rescue of maternal *skn-1* lethality was observed in some *rol*; non-*unc* progeny but not in non-*rol*; non-*unc* animals. SKN-1::GFP expression analyses shown were performed in the *Is007* strain, but essentially the same results were obtained in analyses of *Ex001*, *Ex007*, and *Is001* (data not shown). To analyze expression and localization of SKN-1::GFP in response to oxidative stress, *skn-1::gfp* transgenic worms were treated as described above for the *gcs-1::gfp* expression studies. In addition, for exposure to sodium azide animals cultured at 20°C were placed upon a 2% agarose pad on a slide in M9 either with or without 50 mM sodium azide, then covered with a slip and examined by fluorescence microscopy.

These worms were scored for presence of SKN-1::GFP in intestinal nuclei 5 minutes later. For photography, worms were immobilized either 2 mM Sodium Azide (Fig. 2, 3) or 2 mM Levamisole (Fig. 5). These treatments did not stimulate either GCS-1::GFP induction or SKN-1::GFP relocalization during the times examined (data not shown). No immobilization agent was used in the experiments shown in Tables 2 and 3. To discriminate intestinal autofluorescence from SKN-1::GFP epifluorescence, a triple band emission filter set (Chroma 61000) was used in conjunction with a narrow band excitation filter (484/14 nm). This combination allowed autofluorescence to be detected as yellow/orange fluorescence deriving from a combined green and red signal, while GFP remained green. Worms that carried *skn-1::gfp*, *gcs-1::gfp* and *gcs-1::gfp* mutant transgenes were incubated with 50 µg/ml DiI (Molecular Probes) in M9 for 3 hours at 20°C, then transferred to fresh plates for 1 hour to destain, and examined under the fluorescence microscope. The ASI chemosensory neurons were identified by according to their intensity of DiI labeling and location relative to other DiI-labeled cells.

DNA binding assays

Full-length SKN-1 and the SKN domain were expressed by in vitro translation (Promega) as described previously (Carroll et al., Genes Dev. 11: 2227-2238 (1997)). Oligonucleotide probes were end-labeled using Klenow and α-^{32}P-labelled dATP and CTP, then purified using QIAquick Kit (Qiagen). EMSAs were performed essentially as described in Blackwell et al. (Science 266: 621-628 (1994), with labeled probes present at 2.5×10^{-9} M.

Constitutive and inducible Phase II detoxification gene activation by SKN-1

Vertebrate Nrf proteins induce expression of Phase II detoxification enzyme genes by binding to the characteristic antioxidant response element (ARE) in their promoters (Fig. 1C) (Hayes et al. Cancer Lett 174: 103-113 (2001)). A search was performed for SKN-1 binding sites within the predicted promoters of *C. elegans* orthologs of these oxidative stress resistance genes. The SKN-1 binding site preference and the ARE are distinct but not mutually exclusive (Fig. 1C). A predicted SKN-1 site should appear randomly every 2048 bp, but between two and four SKN-1

sites are present within 1 kb upstream of multiple *C. elegans* genes that encode predicted Phase II detoxification enzymes, including γ-glutamine cysteine synthase heavy chain (GCS(h)), glutathione synthetase, and four glutathione S-transferase (GST) isoforms (Table 1). In vertebrates each of these genes is activated by Nrf proteins (Id.). SKN-1 sites or variants that differ at only one AT-rich region position are similarly present 5' of the Nrf target *NADH quinone oxidoreductase*, the catalase *ctl-1*, and superoxide dismutases (*sod-1, -2* and *-3*) (see Table 1, below).

Table 1. Predicted SKN-1 binding sites upstream of *C. elegans* oxidative stress resistance genes

Enzymes	Gene or ORF	Location[a]	Direction	Sequence
γ-glutamyl-cysteine synthetase heavy chain (GCS(h))	*gcs-1*	-121	→	TTTATCAT
		-316	←	ATGACTTA
		-607	←	ATGACAAT
Glutathione synthetase	M176.2	-137	→	TTTGTCAT
		-169	←	ATGACAAA
		-243	→	TTTATCAT
		-378	←	ATGATTTT
NADH quinone oxidoreductase	F39B2.3	-469	→	GTTATCAT
		-518	←	ATGACAAT
Glutathione S-transferase	R03D7.6	-149	←	ATGACAAT
		-282	←	ATGATTTT
		-302	←	ATGACATT
		-947	←	ATGATTTT
	F35E8.8	-94	←	ATGACAAT
		-240	←	ATGATAAT
	F11G11.2	-133	←	ATGACAAA
		-391	→	CTTATCAT
	K08F4.7	-83	←	ATGACATT
		-157	→	TTTGTCAT
Superoxide dismutase	*sod-1*	-64	→	ATAATCAT
	sod-2	-191	→	TGTATCAT
		-363	←	ATGACAAT
		-959	→	AGAATCAT
		-980	→	AGAATCAT
	sod-3	-287	→	TAAATCAT
Catalase	*ctl-1*	-880	→	ATGATCAT
		-978	→	GTCATCAT
		-997	→	CTTATCAT

The SKN-1 binding consensus is shown in Figure 1C. [a]The A within the translation initiation codon is designated as base 1.

Presence of SKN-1 site clusters upstream of multiple *C. elegans* Phase II detoxification genes is consistent with SKN-1 functioning analogously to Nrf proteins. To confirm this, whether SKN-1 is required to express the Phase II gene *gcs-1* (Table 1) was investigated. *gcs-1* is the *C. elegans* ortholog of *GCS(h)*, a representative and well-characterized Nrf protein target gene that in yeast is regulated by Yap1p and Pap1p (Id., and Toone et al., Curr Opin Genet Dev 9: 55-61 (1999)). The GCS(h) enzyme is important for oxidative stress resistance because it is rate-limiting for glutathione synthesis.

gcs-1 expression in *C. elegans* was investigated using a transgene that included the predicted *gcs-1* promoter, along with the 17 amino terminal GCS-1 amino acids fused to green fluorescent protein (GFP). This promoter segment contained three consensus SKN-1 binding sites, and corresponded to the intervening sequence between *gcs-1* and the nearest upstream gene (data not shown). With this strategy, it was possible to analyze *gcs-1* expression independently of GCS-1protein stability. In a wild-type background, during larval and adult stages GCS-1::GFP was readily detectable in the pharynx, and in nearby cells that appeared to be neurons (Figs. 2A and B). By soaking *gcs-1::gfp* lines in DiI, a dye that fills amphid sensory neurons (Herman et al., Nature 348:169-171 (1990)), it was determined that two GCS-1::GFP-expressing cells located adjacent to the posterior pharynx correspond to the ASI chemosensory neurons (Fig. 2C and 2D), which prevent constitutive entry into the dauer diapause state (Ren et al. 1996; Schackwitz et al. 1996). GCS-1::GFP expression was also apparent anteriorly and posteriorly in the intestine (Figs. 2A and 2B).

In vertebrates, oxidative stress induces Phase II gene expression through an Nrf2-dependent pathway in the intestine and liver (Itoh et al. 1997; Hayes and McMahon 2001). Similarly, stimuli that cause oxidative stress dramatically increased GCS-1::GFP expression in the *C. elegans* intestine (Figs. 2E and 2F, and see Table 2, below). This response was triggered by both heat and the herbicide paraquat (methyl viologen), which generates intracellular superoxide anions. To investigate the involvement of *skn-1* in *gcs-1* expression, we introduced the *gcs-1::gfp* transgene into the *skn-1(zu67)* background, the *skn-1* allele that is associated with the most severe

embryonic phenotype (Bowerman et al. *Cell* 68: 1061-1075 (1992)). Under both normal and oxidative stress conditions, in *skn-1(zu67)* homozygotes GCS-1::GFP was apparent at wild-type levels in the pharynx, but was otherwise undetectable (Figs. 2G-2L), indicating that *skn-1* is essential for both constitutive and inducible *gcs-1::gfp* expression outside of the pharynx.

Promoter mutagenesis identified discrete elements that are required for these *skn-1*-
dependent and –independent *gcs-1* expression patterns. Pharyngeal GCS-1::GFP expression was
abolished by removal of the distal *gcs-1* promoter region (*gcsΔ2::gfp*; Figs. 3A and 3B), which lacks SKN-1 binding sites but contains consensus sites for the pharyngeal transcription factors PEB-1 and PHA-4 (Thatcher et al. *Dev Biol* 229: 480-493 (2001); Gaudet et al., *Science* 295: 821-825 (2002)) (data not shown). The remaining proximal 682 bp of the *gcs-1* promoter included the three predicted SKN-1 binding sites, and was sufficient for appropriate GCS-1::GFP expression in the intestine and ASI neurons (*gcsΔ2::gfp*, Figs. 3A, 3B, Table 2). Constitutive and stress-induced GCS-1::GFP expression within the intestine and ASI neurons did not require SKN-1 binding sites 1 or 2 individually, but was abolished by alteration of site 3 (*gcs 2;mut3::gfp*; Figs. 3A and 3B).

Table 2. Induction of GCS-1::GFP expression in the intestine by oxidative stress

	gcs-1::gfp				*gcsΔ2::gfp*			
Inducer	low	medium	high	N	low	medium	high	N
Control	90.8%	7.9%	1.3%	76	88.2%	10.3%	1.5%	68
Heat Shock	10.5%	72.4%	17.1%	76	0.0%	14.0%	86.0%	86
Paraquat	14.5%	67.1%	18.4%	76	21.3%	65.6%	13.1%	61

A representative set of experiments involving a mixed population of L2-young adult worms is shown, from which percentages of animals in each expression category are listed. Induction of GCS-1::GFP expression was comparable among the different developmental stages analyzed. "Low" refers to animals similar to that in Fig. 2A, in which intestinal GCS-1::GFP was apparent at modest levels anteriorly, or anteriorly and posteriorly. "High" indicates that a GCS-1::GFP was present at high levels anteriorly and detectable throughout most of the intestine, as in Fig. 2F. "Medium" refers to animals in which GCS-1::GFP was present at high levels anteriorly as in Fig. 2F and possibly posteriorly, but was not detected in between. N indicates numbers of animals analyzed from each transgenic strain.

Remarkably, SKN-1 binding site 3 is located within a 42 bp *gcs-1* promoter element that is similar to a composite motif through which SKN-1 activates *med-1* and *med-2* in the embryo (Figs. 1A and 3C) (Maduro et al. Mol. Cell 7: 475-485 (2001)). The conservation between these *gcs-1* and *med* promoter elements is particularly striking because they are located at identical distances from their respective translation starts, but contain different numbers of SKN-1 sites (Figs. 3C). In an electrophoretic mobility shift assay (EMSA), full-length SKN-1 and the 85 amino acid SKN-1 DNA binding domain (SKN Domain) (Blackwell et al. Science **266**: 621-628 (1994)) each bound sequence-specifically to SKN-1 binding site 3 in the context of this *gcs-1* promoter element (Fig. 4). These SKN-1 proteins bound with high affinity to an oligonucleotide that corresponds to this composite element (Wild type; Figs. 4A and 4B, lanes 2-5), but not to an analogous probe in which SKN-1 site 3 had been altered as in the inactive *gcsΔ2; mut3::gfp* transgene (Fig. 3A; Mutant, Figs. 4A and 4B, lanes 7-10). Binding of these SKN-1 proteins to the Wild type

probe was also competed much more effectively by unlabeled Wild type than Mutant DNA (Figs. 4A and B, lanes 11-20). Further supporting the importance of this *gcs-1* promoter element, a 163 bp fragment that includes it provides significant GCS-1::GFP expression in the intestine, but 5' truncation within this sequence inactivates the promoter (*gcsΔ4::gfp* and *gcsΔ5::gfp*, Fig. 3A). It is evident that binding of SKN-1 to site 3 is required for *gcs-1* expression in the intestine and ASI neurons.

SKN-1 expression and accumulation in intestinal nuclei in response to oxidative stress

To determine whether SKN-1 is present in tissues where it is required for *gcs-1::gfp* expression, expression of a transgene in which GFP is fused to the C-terminus of full-length SKN-1 (SKN-1::GFP; Fig. 5A) was analyzed. Although maternal *skn-1::gfp* expression was not readily detectable because of germline transgene silencing (Kelly et al., *Genetics* 146:227-238 (1997)), at a low frequency this transgene rescued the embryonic defect in *skn-1(zu67)* homozygotes (data not shown), indicating that this SKN-1::GFP fusion protein is functional.

In the embryo, antibody staining previously revealed presence of maternal SKN-1 in nuclei through the eight-cell stage, then detected zygotically expressed SKN-1 in only about 15% of late-stage embryos that had ceased dividing (Bowerman et al. *Cell* 74: 443-452 (1993)). Nuclear SKN-1::GFP was uniformly detected in intestinal precursors beginning at the 50-100 cell stage (Fig. 5B), then in both the intestine and hypodermis (Fig. 5C), indicating that SKN-1 is expressed zygotically earlier than it is detectable by antibody staining. In late-stage embryos SKN-1::GFP was also present in intestinal nuclei but not in the hypodermis (Fig. 5D), suggesting that hypodermal *skn-1* expression may be maintained by a region located outside of this transgene.

In contrast to the embryo, in larvae and young adults SKN-1::GFP was usually present at very low levels in intestinal nuclei (Fig. 5F, and see Table 3, below). SKN-1::GFP was readily detectable in the ASI neurons, where *gcs-1::gfp* was constitutively expressed (Figs. 5E and 5F), but not in other cells in the head where GCS-1::GFP expression appeared to be *skn-1*-dependent (Figs. 2B and 2H). The latter *skn-1* dependence might be indirect, or derived from low level SKN-1 expression or distant

*skn-1*regulatory regions. The finding that SKN-1::GFP is present at only modest levels in intestinal nuclei raises the question of how oxidative stress induces *skn-1*–dependent intestinal *gcs-1* expression (Figs. 2, 3, and Table 2, above). In cultured mammalian cells, Nrf2 is stabilized and relocalized from the cytoplasm to the nucleus in response to oxidative stress (Itoh et al. *Genes Dev* 13: 76-86 (1999); Sekhar et al. *Oncogene* 21: 6829-6834 (2002); Nguyen et al. *J Biol Chem* 278: 4536-4541(2003); and Stewart et al. *J Biol Chem* 278: 2396-2402 (2003)). A promoter fusion transgene in which only the SKN-1 amino terminus was linked to GFP (SknPro::GFP, Fig. 5A) was constitutively expressed at high levels in all intestinal cells (data not shown), suggesting that SKN-1 expression or localization might also be regulated post-transcriptionally by oxidative stress.

After exposure to either paraquat or heat, neither the location nor intensity of SKN-1::GFP was detectably altered in the ASI neurons, but in a high percentage of animals elevated levels of SKN-1::GFP appeared in intestinal cell nuclei, particularly anteriorly and posteriorly where GCS-1::GFP is most robustly expressed (Figs. 5F and 5G, Table 3). SKN-1::GFP accumulated in intestinal nuclei within 5 min. after treatment with 50 mM sodium azide (Table 3), which induces oxidative stress by blocking mitochondrial electron transport. The rapidity of this last response indicates that in the intestine SKN-1 is constitutively present, but may be diffuse within the cytoplasm and masked by autofluorescence. This accumulation of SKN-1::GFP in intestinal nuclei in response to oxidative stress remarkably parallels the *skn-1*-dependent induction of GCS-1::GFP under similar conditions, supporting the model that SKN-1 activates intestinal *gcs-1* expression directly.

Table 3. Accumulation of SKN-1::GFP in intestinal nuclei in response to oxidative stress

Inducer	low	medium	high	N
Control	78.9%	14.5%	6.6%	76
Heat	5.6%	11.9%	82.5%	143
Paraquat	53.1%	43.8%	3.1%	64
M9, 5 min.	74.7%	17.6%	7.7%	91
50 mM Sodium Azide, 5 min.	0.8%	44.2%	55.0%	120

Mixed-stage L2-young adult transgenic worms were exposed to the indicated conditions. A representative set of experiments is shown, from which percentages of animals in each category are listed. SKN-1 localization patterns did not differ significantly among the different developmental stages examined. M9 refers to the control incubation for the sodium azide experiment. In some animals treated with sodium azide, high levels of nuclear SKN-1::GFP appeared in less than 1 minute (data not shown). "Low" refers to animals in which SKN-1::GFP was barely detectable in all intestinal nuclei, as shown in Fig. 4F. "High" indicates that a very strong SKN-1::GFP signal was present in all intestinal nuclei, as in Fig. 4G. "Medium" refers to animals in which nuclear SKN-1::GFP was present at high levels anteriorly or anteriorly and posteriorly, but was barely detectable midway through the intestine. N indicates numbers of animals analyzed in each category.

SKN-1 accumulation in intestinal nuclei following inhibition of sgg-1 (GSK-3)

To determine the role of sgg-1 (GSK-3) in oxidative stress response, the effect of sgg-1 (GSK-3) inhibition on SKN-1 localization was investigated. When expression of sgg-1 (GSK-3) was reduced using sgg-1 (GSK-3) RNAi, SKN-1::GFP accumulated in intestinal nuclei (Fig. 37A). Further, GCS-1::GFP was induced moderately in sgg-1 (RNAi) worms (Fig. 37B). These findings indicate that sgg-1 (GSK-3) inhibits constitutive SKN-1 nuclear accumulation and induction of its target gene, gcs-1.

GSK-3 directly regulates SKN-1

To determine whether GSK-3 directly regulates SKN-1, seven predicted GSK-3 sites in SKN-1 were substituted with alanine (Figure 38). Transgenic worms expressing alanine-substituted SKN-1 were analyzed for SKN-1::GFP localization.

Alanine substitution resulted in nuclear accumulation of SKN-1, especially in transgenic worms expressing the SKN-1 mutant S393A.

Phosphorylation of SKN-1 by GSK-3 was also investigated. Phosphorylation by GSK-3 required priming phosphorylation at a serine or threonine residue located four positions carboxyl-terminal to the GSK-3 site (Fig. 39). GSK-3 then phosphorylates SKN-1 sequentially in the amino-terminal direction.

skn-1 required for oxidative stress resistance and normal longevity

Whether *skn-1* mutants are abnormally sensitive to oxidative stress was investigated. *skn-1(zu67)* homozygotes produce normal numbers of offspring with normal timing, and as young adults are not obviously distinguishable in morphology from wild-type (data not shown). Two different *skn-1* mutant alleles were associated with markedly decreased survival in the presence of paraquat however, indicating that *skn-1* mutants are sensitive to oxidative stress (Fig. 6A).

Further, whether *skn-1* homozygotes live as long as wild type was investigated. Both the mean and maximum lifespans of *skn-1(zu67)* and *skn-1(zu129)* homozygotes were reduced by 25-30% (Fig. 6B), indicating that SKN-1 is required for normal longevity.

A conserved postembryonic function for SKN-1 in oxidative stress resistance

The *C. elegans* developmental specification protein SKN-1 also mediates a conserved response to oxidative stress. SKN-1 functions similarly to bZIP proteins that regulate Phase II detoxification genes in vertebrates (Nrf1, 2) and yeast (Yap1p, Pap1p). SKN-1 activates a conserved Phase II gene in the intestine and ASI neurons (Figs. 2, 3, 5), SKN-1 binding sites flank *C. elegans* orthologs of additional Nrf target genes (Table 1), and *skn-1* mutants are sensitive to oxidative stress (Fig. 6A). The accumulation of SKN-1 in intestinal nuclei in response to oxidative stress (Fig. 5G, and Table 3) may parallel nuclear accumulation of Nrf proteins, Yap1p, and Pap1p, under these conditions (Itoh et al. *Genes Dev* 13: 76-86 (1999); Toone et al. *Oncogene* 20: 2336-2346 (2001); and Delaunay et al., *Cell* 111: 471-81 (2002)). The intestinal abnormalities in *skn-1(zu67)/nDf41* larvae (Bowerman et al. *Cell* 68: 1061-1075

(1992)) could involve oxidative stress, because 10-20% of *gcs-1(RNAi)* animals also die as
larvae with abnormal intestines (data not shown).

These parallels between SKN-1 and Nrf proteins are surprising because the mechanism through which SKN-1 binds DNA is both unique and highly divergent (Blackwell et al. *Science* 266: 621-628 (1994)). SKN-1 and Nrf proteins are most similar within the 14 amino acid DIDLID transactivation element (Fig. 1B) (Walker et al. *J. Biol. Chem.* 275: 22166-
22171 (2000)).

While *gcs-1* expression in the intestine is induced by SKN-1 in response to stress, presence of nuclear SKN-1 allows *gcs-1* to be expressed constitutively in the ASI neurons, and *gcs-1* expression is *skn-1*-independent in the pharynx (Fig. 2). In metazoans, Phase II genes thus can be activated through distinct pathways that may be important for functions of different tissues. For example, the finding that *skn-1* functions constitutively in the ASI neurons, which inhibit dauer entry, suggests that although *skn-1(zu67)* homozygotes can enter the dauer stage (data not shown), *skn-1* or oxidative stress might influence regulation of this process.

The lifespan reduction that observed in *skn-1* mutants (25-30%, Fig. 6B) is comparable to that reported in *daf-16* mutants (20%) (Kenyon et al. *Nature* 366: 461-464 (1993); and Lee et al. *Curr Biol* 11: 1950-1957 (2001)). In *C. elegans,* aging involves pleiotropic changes that vary among individuals, and mutations that influence lifespan may affect aging of some tissues more than others (Garigan et al. *Genetics* 161: 1101-1112 (2002); and Herndon et al. *Nature* 419: 808-814 (2002). Just before death the anterior intestine and posterior pharynx degenerated more frequently in *skn-1* animals than wild type (data not shown), a finding that may reflect aging but does not exclude the possibility of an additional defect. At one week after hatching, small cavities and apparent yolk droplets appeared in the heads of many *skn-1* but not wild type animals (data not shown). These changes in the head region are typical of aging *C. elegans* (Garigan et al. *Genetics* 161: 1101-1112 (2002); Herndon et al. *Nature* 419: 808-814), suggesting that *skn-1* mutants age prematurely. Some mechanisms that regulate *C. elegans* lifespan have been shown to influence lifespan in higher metazoans (Clancy et al. *Science* 292: 104-106 (2001); Finch et al.

Annu Rev Genomics Nature 421: 182-187 (2003)). The observation that normal *C. elegans* longevity requires *skn-1* is consistent with other associations between oxidative stress resistance and lifespan, and suggests that the conserved oxidative stress resistance pathway regulated by SKN-1 might influence longevity in other species.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.